APPENDIX D

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Biological Activity of Follistatin Isoforms and Follistatin-Like-3 Is Dependent on Differential Cell Surface Binding and Specificity for Activin, Myostatin, and Bone Morphogenetic Proteins

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Follistatin (FST) and FST-like-3 (FSTL3) are activin-binding and neutralization proteins that also bind myostatin. Three FST isoforms have been described that differ in tissue distribution and cell-surface binding activity, suggesting that the FST isoforms and FSTL3 may have some nonoverlapping biological actions. We produced recombinant FST isoforms and FSTL3 and compared their biochemical and biological properties. Activin-binding affinities and kinetics were comparable between the isoforms and FSTL3, whereas cell-surface binding differed markedly (FST288 > FST303 > FST315 > FSTL3). Inhibition of endogenous activin bioactivity, whether the FST isoforms were administered endogenously or exogenously, correlated closely with surface binding activity, whereas neutralization of exogenous activin when FST and FSTL3 were also exogenous was consistent with their equivalent activin-binding affinities. This difference in activin inhibition was also evident in an *in vitro* bioassay because FST288 suppressed, whereas FST315 enhanced, activin-dependent TT cell proliferation. Moreover, when FSTL3, which does not associate with cell membranes, was expressed as a membrane-anchored protein, its endogenous activin inhibitory activity was dramatically increased. In competitive binding assays, myostatin was more potent than bone morphogenetic proteins (BMPs) 6 and 7, and BMPs 2 and 4 were inactive in binding to FST isoforms, whereas none of the BMPs tested competed with activin for binding to FSTL3. Neutralization of exogenous BMP or myostatin bioactivity correlated with the relative abilities of the isoforms to bind cell-surface proteoglycans. These results indicate that the differential biological actions among the FST isoforms and FSTL3 are primarily dependent on their relative cell-surface binding ability and ligand specificity. (*Endocrinology* 147: 3586–3597, 2006)

FOLLISTATIN (FST) IS AN extracellular regulatory protein for activin and related TGFβ superfamily members that acts via high-affinity, nearly irreversible binding to prevent activin from accessing its receptor (reviewed in Refs. 1 and 2). Although originally isolated from gonadal fluids and thought to act as an endocrine regulator of pituitary FSH release, the subsequent identification of FST mRNA and protein in numerous adult and embryonic tissues as well as the colocalization of FST with activin A in many tissues supports the currently held view that FST acts largely in an autocrine/paracrine manner (reviewed in Ref. 3).

FST has a number of putative physiological roles, including regulating pituitary FSH production (4), ovarian follicle maturation (5), spermatogenesis (6), liver homeostasis (7), wound repair (8), and response to inflammatory stimuli (9). Moreover, disruption of the mouse *Fst* gene resulted in developmental abnormalities including loss of hair, weakened musculature, XX sex reversal, and early neonatal death, demonstrating that FST is required for normal mammalian development (10, 11). However, the identification of precise activities and mechanisms of action for FST in both embryos and adults has been hampered by the fact that multiple isoforms of FST are produced from the *FST* gene, each with potentially distinct activities. The primary FST transcript undergoes alternative splicing to produce mRNAs that code for two FST proteins, termed FST288 and FST315 (12). The FST315 isoform contains all six exons, whereas the FST288 splice variant is missing exon 6, which codes for the acidic C-terminal tail. A third isoform, FST303, appears to arise from proteolytic cleavage of the FST315 C-terminal tail between residues 300 and 303 (13). All three isoforms contain a region of basic residues known as the heparin-binding sequence (HBS), which is essential for binding to cell-surface heparin-sulfated proteoglycans (14–17). However, it has been proposed that the acidic tail in FST315 interacts with the basic residues within the HBS, thereby suppressing the cell-surface binding activity of FST315. FST303, with its shortened tail, has cell-surface binding activity intermediate between FST315 and FST288 (13). These biochemical distinctions suggest that each isoform may be responsible for different subsets of biological activities depending on their degree of cell-surface localization and subsequent compartmentalization within the body, a concept supported by the finding that FST315 is the predominant circulating FST isoform in human serum (18), whereas ovarian follicular fluid contains primarily FST303 (14). Nevertheless, differential biological activity as well as the underlying mechanisms among the FST isoforms remain to be fully elucidated.

FST-like-3 (FSTL3), also known as FST-related gene

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Abbreviations: BMP, Bone morphogenetic protein; FBS, fetal bovine serum; FST, follistatin; FSTL3, follistatin-like-3; HBS, heparin-binding sequence.

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(FLRG) (19) and FST-related protein (FSRP) (20), shares substantial structural and functional homology with FST (21), including inhibition of activin bioactivity *in vivo* (22). Importantly, FSTL3 does not have an HBS, cannot bind to cell-surface proteoglycans (23), and is a weak antagonist of endogenous (autocrine) activin despite being only slightly less potent in neutralizing exogenous (endocrine/paracrine) activin (23). These distinctions between FSTL3 and FST support the concept that the presence of a functional HBS and resultant cell-surface binding is a critical biochemical determinant for endogenous activin inhibition.

In addition to activin, FST and FSTL3 bind other members of the TGFβ superfamily, including myostatin (24, 25) and some bone morphogenetic proteins (BMPs) (26, 27). FSTL3 was recently identified as the circulating binding protein for myostatin in mice and humans (24), suggesting that FSTL3 may have an important role in regulating muscle development and/or adult muscle mass. These studies indicate that both FST and FSTL3 likely play important roles in regulating the physiological and homeostatic activities of activin and related TGFβ superfamily ligands and that the physiological significance of these regulatory interactions, as well as quantitative differences in specificity among the binding proteins, remains to be determined systematically.

Given the different biochemical properties and compartmentalization of the FST isoforms and FSTL3, we hypothesized that these proteins will also have distinct mechanisms of action and ligand selectivity that will ultimately determine their range of physiological actions *in vivo*. To explore molecular mechanisms responsible for this differential bioactivity, we produced recombinant FST isoforms and investigated their differential biochemical and biological activities compared with FSTL3. Our results indicate that the biological activities of the FST isoforms and FSTL3 are determined primarily by their differential cell-surface binding and ligand specificity and further support the concept that FST isoforms may have distinct physiological roles *in vivo*.

Materials and Methods

Reagents

Recombinant human activin A, BMP2, and BMP4 were purchased from R&D Systems (Minneapolis, MN). Myostatin was purchased from Cell Signaling (Canton, MA), whereas BMP6 and -7 (OP1) were a gift from Creative Biomolecules (Cambridge, MA).

Preparation of recombinant tagged FST and FSTL3 proteins

Human FST288, FST315, and FSTL3 (the latter a gift from Millennium Pharmaceuticals, Cambridge, MA) coding sequences were subcloned into pCDNA3.1Myc-His (Invitrogen, Carlsbad CA). The FST303 expression construct was prepared from the FST315 cDNA by deleting all sequence 3′ to codon 303 up to the start of the Myc-His tag using standard PCR methods. The resulting C-terminal Myc-His-tagged cDNA constructs were transfected into HEK-293-F cell suspension cultures in Freestyle serum-free medium (Invitrogen) as described (28). Recombinant proteins were purified by nickel-Sepharose affinity chromatography (QIAGEN, Valencia, CA) and concentrated by centrifugal dialysis into Dulbecco's PBS.

Quantitation of secreted proteins

FST concentrations in media and concentrated affinity-purified eluates were established by two independent immunological assays: 1) a two-site solid-phase immunochemiluminescent assay (29) and 2) a solution-phase assay directed toward the C-terminal Myc tag (28). The concentrations obtained by the two methods were usually in good agreement for all FST preparations. FSTL3 preparations were quantified by the Myc RIA. Concentration and purity of affinity-purified proteins were verified by SDS-PAGE and silver staining (Bio-Rad Laboratories, Hercules, CA) or Western blot as described below.

Western blot analysis

Proteins were separated on 12% Tris-HCl Ready-Gel system (Bio-Rad), transferred to polyvinylidene difluoride membrane (Bio-Rad), blocked in 10% nonfat dry milk, and probed with anti-Myc (1 μg/ml, clone 4A6; Upstate Biotechnology, Lake Placid, NY) and goat antimouse horseradish peroxidase-conjugated (1:7500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Immunoreactivity was visualized using Western Lightning chemiluminescence reagent (PerkinElmer, Boston, MA).

Iodination

Human activin A was iodinated by the lactoperoxidase method and purified by electrophoresis as described previously (30).

Binding to cell-surface heparan-sulfate proteoglycans

COS cells were cultured in DMEM supplemented with 10% FBS (Invitrogen) and plated into 24-well plates and cultured to confluence. FST isoforms or FSTL3 was added at 50 ng/ml for 1 h at 25 C in fresh medium containing 0.1% BSA, washed, and replaced with medium containing 75,000 cpm [^{125}I]activin A alone or together with 10 μg/ml heparan sulfate (Sigma Chemical Co., St. Louis, MO), which displaces FST-activin complexes. After 1 h incubation at 25 C, cells were rinsed, harvested, and counted to detect FST-bound activin.

Activin-binding kinetics

Binding kinetics of FST isoforms to radiolabeled activin was determined by solid binding assay as described (31) using 25 ng/well of each isoform passively adsorbed to microtiter plates. After blocking the wells with 3% BSA/0.01% Tween in 10 mM PBS, 75,000 cpm radiolabeled activin was added. To determine the association rate, the binding reactions were terminated at the indicated times, the wells were washed, and bound radioligand was counted in a γ-counter. For dissociation measurements, the binding reactions were allowed to continue to steady state (3 h), after which unbound radioligand was removed and 100-fold excess of unlabeled ligand was added. At the indicated times, the wells were washed and counted as before. Maximum binding in a typical experiment reached 10–15% of total counts. Observed association rate constants (K_{on}) and dissociation rate constants (K_{off}) were calculated by fitting an exponential association equation and exponential decay model, respectively, using Prism4 (GraphPad Software Inc., San Diego, CA). Binding kinetics was assayed in at least three independent experiments for each isoform.

FST and FSTL3 specificity assays

Nonequilibrium competitive binding assays were used to determine isoform specificity as described (31). Microtiter plates were prepared with FST isoforms or FSTL3 as described above. Unlabeled activin A or heterologous competitors at doses indicated in the figures were individually mixed with 75,000 cpm [^{125}I]activin A in 150 μl assay buffer for 1 h. This nonequilibrium assay format was used to compensate for the heavily favored and nearly irreversible activin-binding kinetics (31) that would have displaced competitors if the assay had been allowed to reach steady state (>2 h). At the end of the 1-h incubation, the wells were washed and counted. In a typical experiment, about 10–15% of the added total counts were bound in the absence of unlabeled competitor. Resulting inhibition curves were analyzed using the four-parameter logistic model. Each ligand was assayed in at least three independent experiments.

Cell culture and reporter assays

The capacity of FST isoforms and FSTL3 to inhibit bioactivity of activin and related TGFβ family ligands was determined by reporter assays in human HepG2 cells (for activin A and BMPs), and human embryonic kidney (HEK) 293 cells (for activin A and myostatin), because myostatin responses were relatively weak in HepG2 cells. HepG2 cells were maintained in MEM supplemented with Earle's salts, nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum (FBS) (Life Technologies, Inc., Rockville, MD). Cells were cultured in 24-well trays and transiently transfected with the smad1/5-responsive reporter BRE-Luc (32) for BMP activity or the smad2/3-responsive reporter CAGA-luc (33) for activin A activity, along with pRL-TK (Promega Corp., Madison, WI) and the indicated doses of FST isoform cDNAs (for experiments examining bioactivity of endogenous FST or FSTL3) or empty vector, using Lipofectamine 2000 (Invitrogen). To examine the effect of isoforms on endogenous activin, 1 ng pINHβA cDNA construct (kindly provided by Genentech Inc., San Francisco, CA) was included in the transfection mix. After 20 h, 0.2 nM activin A (for experiments on exogenous activin) was added, or was premixed (60 min at 25 C) with increasing amounts of purified FST isoforms or FSTL3 (for exogenous FST/FSTL3 experiments). After 16 h of treatment, the cells were washed, lysed with Passive lysis buffer (Promega) and assayed for luciferase activity using the dual luciferase reporter assay kit (Promega). Interwell variations in transfection efficiency were corrected by normalizing to Renilla luciferase activity. For each ligand, experiments were repeated at least three times, and the mean and SE of representative experiments are reported.

HEK 293 cells were maintained in RPMI 1640 medium containing 10% FBS (Life Technologies). Transient transfections and reporter assays were performed as described above except for using Effectene as the transfection reagent (QIAGEN) and included the CAGA-luc reporter and pRL-TK control cDNA, as well as FST or FSTL3 expression constructs and pINHβA cDNA for endogenous FST/FSTL3 or activin experiments, respectively.

To examine the effect of cell-surface association on FSTL3 ability to block endogenous activin, FSTL3 coding sequence without the signal peptide was cloned in frame with the transmembrane domain of pDisplay (Promega) and tested in CAGA-luc reporter assays in HEK 293 as described above.

To verify that transfected FST isoforms and FSTL3 were expressed at similar levels, 10 μl cell extract and 20 μl conditioned medium from the highest-dose wells were subjected to reduced SDS-PAGE and Western analysis as described above.

TT cells transfection and proliferation assays

TT cells (provided by Dr. Aaron Hsueh, Stanford University, Stanford, CA) are a clonal, testicular tumor cell line derived from a p^{53}/α-inhibin subunit-deficient mouse line (34). Cells were cultured in DMEM-Ham's F-12 (1:1) supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, and antibiotics.

TT cells were transfected with expression constructs containing full-length FST288 or FS315 cDNAs (kindly provided by Dr. S. Shimasaki, University of California, San Diego, La Jolla, CA) in pCDNA3 vector (Invitrogen). Stable colonies were isolated and screened for FST secretion using our two-site solid-phase immunochemiluminescent assay for free (unbound to activin) FST as previously described (29). Cellular proliferation was assessed using the CellTiter 96 AQueous One Solution Reagent (Promega) colorimetric assay according to the manufacturer's recommendations.

Generation of recombinant adenovirus and proliferation of infected TT cells

Adenoviruses encoding FST288 and FST315 were constructed using a previously described two-cosmid adenoviral system (35). Briefly, full-length FST288 and FST315 cDNAs were cloned into the pLEP plasmid and then ligated to a cosmid containing the adenoviral genome (pREP7). The ligation product was packaged in phage packaging extracts (MaxPlax; Epicenter Technologies) infected into host bacteria, and hybrid cosmids were selected, amplified, and transfected into HEK 293 cells. High-titer stock solutions of adenovirus were obtained by repeated amplifications in HEK 293 cells.

For cell proliferation studies with FST viruses, early-passage stocks of TT cells were plated into 60-mm culture dishes. After 24 h incubation, 500 μl of HEK 293-conditioned medium containing a high titer of FST288-AdV, FST315-AdV, or empty virus, which contained no cDNA, was added. The next day, the cells were washed and seeded into 96-well plates. Cell proliferation assays were then preformed as described above for transfected cells.

Immunocytochemistry

HepG2 cells were grown on coverslips, fixed in 4% paraformaldehyde in PBS for 20 min, and either left nonpermeabilized or permeabilized with 0.1% Triton X-100. Myc-tagged FST isoforms or FSTL3 (1 μg/ml) was then incubated with cells for 1 h at room temperature in the absence or presence of heparin sulfate (100 μg/ml). After washing, cells were treated with anti-Myc monoclonal 4A6 (Upstate, Charlottesville, VA) and antimouse-tetraethylrhodamine isothiocyanate second antibody (Jackson ImmunoResearch, West Grove, PA).

Statistics

Activin-binding kinetic data were analyzed by one-way ANOVA for differences between isoforms followed by Tukey test. Differences of $P < 0.05$ were considered significant.

Results

Production of recombinant FST isoforms

To compare the biochemical and biological properties of FST isoforms and FSTL3, we produced recombinant FST288, FST303, FST315, and FSTL3 and compared their biochemical and biological properties. All preparations were more than 90% pure when analyzed by silver stain and Western blot analysis (Fig. 1). Under reducing conditions, FST288, FST303, and FST315 migrated at apparent molecular weights (M_r)

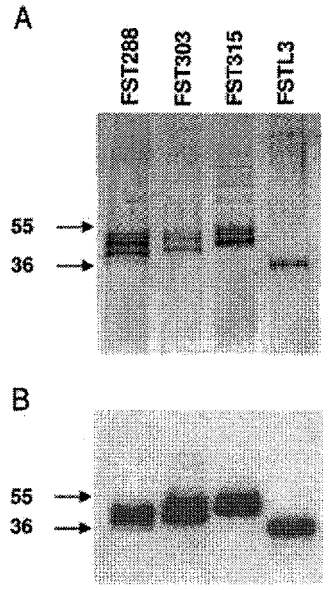

FIG. 1. Analysis of recombinant Myc-tagged FST isoform and FSTL3 proteins. A, Silver stain of FST isoforms and FSTL3 (200 ng/lane) after SDS-PAGE under reducing conditions showing proteins were more than 90% pure; B, Western analysis of similar gel containing 20 ng protein/lane using an anti-Myc antibody, verifying the identity of the nickel-affinity-purified proteins. The recombinant proteins ran as doublets or triplets indicating different degrees of glycosylation with M_r from 38,000–44000 (FST288), 42,000–47,000 (FS303), and 45,000–50,000 (FST315). FSTL3 migrated as a single 36,000 M_r species.

ranging from 38,000–50,000 and appeared as doublets or triplets, whereas purified FSTL3 migrated as a single molecular species at 36,000 M_r. These apparent molecular weights are consistent with previously reported estimates for these proteins, taking into account the size of the Myc-His tag and variable degrees of *n*-glycosylation. Moreover, relative concentrations of the purified proteins were verified by Western blot with anti-Myc antibody because all four proteins could be simultaneously analyzed with a single antibody. These Myc-tagged proteins had identical activin binding, cell-surface binding, and biological activity to untagged proteins (data not shown).

Activin-binding affinities of FST isoforms

It has been suggested that the FST isoforms have differential activin-binding affinity (14, 36). We therefore evaluated the activin-binding kinetics of the FST isoforms and FSTL3 using a solid-phase binding assay with radiolabeled activin A. All FST isoforms and FSTL3 demonstrated rapid activin binding (Fig. 2A) with nearly undetectable dissociation rates (Fig. 2B), similar to previous observations for untagged FST288 and FSTL3 (21). The different levels of total activin binding among the isoforms may represent differential isoform association with the solid-phase support. Nevertheless, dissociation constants determined as K_{off}/K_{on} demonstrated high-affinity activin binding that was not significantly different among the four proteins (Table 1). Thus, there does not appear to be a significant difference in direct binding to soluble activin between the FST isoforms.

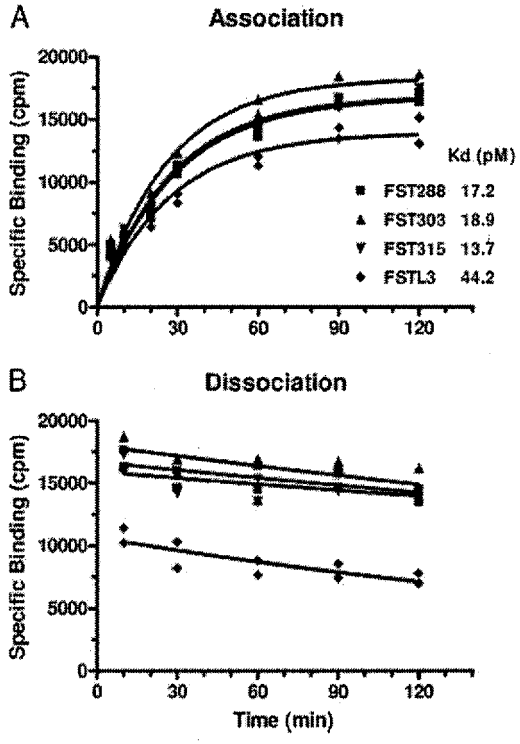

FIG. 2. Activin-binding kinetics of FST isoforms and FSTL3. Purified FST or FSTL3 isoforms were adsorbed to 96-well plates, blocked, and then incubated with 75,000 cpm [^{125}I]activin in the absence or presence of excess unlabeled activin. A, Reaction was terminated at indicated times, wells were washed, and then bound activin was determined. B, After binding reached steady state (3 h), excess unlabeled activin was added for indicated times, after which wells were washed and counted. Radiolabeled activin bound to FST isoforms and FSTL3 with a similar fast association rate but negligible dissociation rate. Affinities, calculated from the on and off rate constants, were not statistically different.

Differential association with cell-surface proteoglycans

Another mechanism whereby FST isoforms might differentially regulate activin action is through distinct association kinetics with cell-surface proteoglycans, an activity that has been shown to be modulated by the presence and length of the acidic C-terminal domain on FST315 (13). We first assessed the relative capacity of our isoform preparations to bind cell-surface proteoglycans using [^{125}I]activin as an indirect measure of FST cell-surface binding. FST288-treated COS cells bound 8-fold more radiolabeled activin compared with untreated cells, and this radioactivity could be removed by heparan sulfate treatment, indicating that the activin was bound to proteoglycan-associated FST288 (Fig. 3A). FSTL3 did not bind to cell-surface proteoglycans as previously reported (23). Only a small fraction of FST315, which contains a full-length C-terminal tail, bound to cell-surface proteoglycans (<50% above basal), whereas FST303, with about half the C-terminal tail removed, bound 5-fold more activin compared with basal but less than half of FST288, indicating an intermediate cell-surface binding ability.

To directly assess binding of FST isoforms to the cell surface, immunocytochemistry was used on nonpermeabilized cells treated with individual FST isoforms or FSTL3. FST288 bound quite well to plasma membranes, whereas substantially less FST303, and no FST315 or FSTL3, was detectable (Fig. 3B, *row* A). All FST was displaced in the presence of heparin (Fig. 3B, *row* B), indicating that this cell-surface-associated FST was reversibly bound to proteoglycans. Interestingly, when cells were first permeabilized, substantially more FST288 was detectable bound to membranes. This staining appeared to be intracellular as well as on the plasma membrane (Fig. 3B, *row* C), and the FST288 was again displaceable by heparin (Fig. 3B, *row* D), suggesting that FST288 may bind to membrane proteoglycans both outside and inside cells. Permeabilized cell staining was much reduced for FST303 and absent for FST315, consistent with binding to nonpermeabilized cells. However, slight staining was observed for FSTL3 in permeabilized cells that was not displaced by heparin (Fig. 3B, *rows* C and D), suggesting that Triton permeabilization treatment may have exposed a binding moiety for FSTL3 that is not used by FST isoforms. Taken together, these results demonstrate that the FST isoforms and FSTL3 have distinct capacities to interact with cell-surface proteoglycans and accumulate at the cell surface in correspondence with the presence of an HBS and the length of the C-terminal tail.

Differential inhibition of exogenous vs. endogenous activin by FST isoforms

To determine whether differences in cell-surface binding translated into differential bioactivity, the FST isoforms and FSTL3, added exogenously or expressed endogenously, were

TABLE 1. Activin binding on rates, off rates, and dissociation constants of FST isoforms

	FST288	FST303	FST315	FSTL3
K_{on} (M^{-1} min^{-1}) $\times 10^7$	7.46 ± 1.5	6.21 ± 1.1	5.51 ± 0.99	5.87 ± 1.3
K_{off} (min^{-1}) $\times 10^{-3}$	1.52 ± 0.12	1.81 ± 0.36	1.45 ± 0.21	2.48 ± 0.76
K_d ($M \times 10^{-11}$)	2.36 ± 0.58	3.02 ± 0.46	2.87 ± 0.52	3.93 ± 0.47

Shown are mean and SEM of four independent determinations. No significant differences were found among the isoforms by ANOVA.

examined for their ability to inhibit endogenous *vs.* exogenous activin in HepG2 hepatoma cells, which have a robust response to activin using the CAGA-luc reporter but do not express FST or detectable activin. When individual FST isoforms or FSTL3 was added with activin as exogenous treatments to the cells, the isoforms were equivalent in their ability to neutralize this activin (Fig. 4A), consistent with their comparable affinities for activin (see Table 1). In contrast, when the FST isoforms and FSTL3 were transfected, *i.e.* produced endogenously, but the activin treatment was exogenous, FST288 was clearly superior among the isoforms in inhibiting exogenous activin (Fig. 4B), and the order of their activity is consistent with the order of cell-surface association (see Fig. 3A). This pattern was also observed when FST

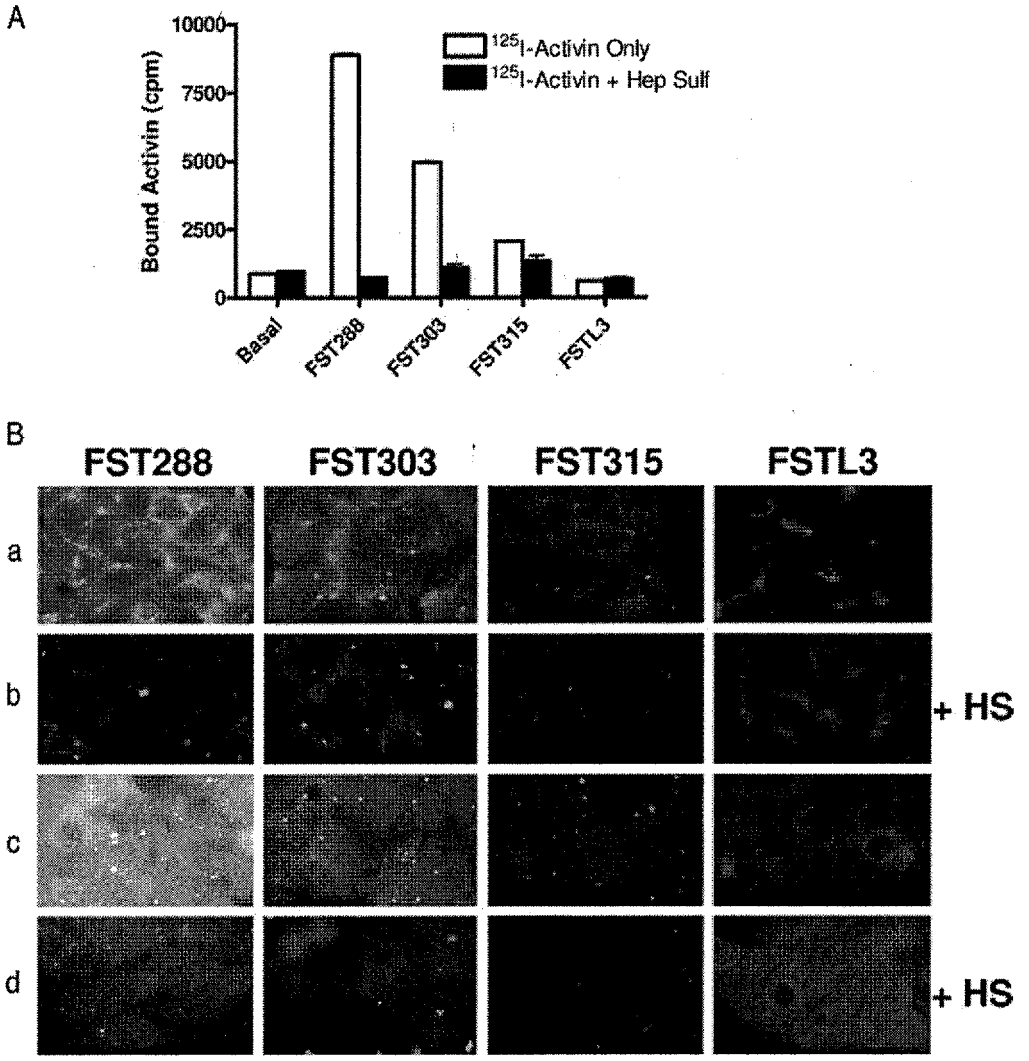

FIG. 3. Association of FST isoforms and FSTL3 with cell-surface proteoglycans. A, COS cells were treated with equal amounts (50 ng/ml) of FST isoforms or FSTL3 preps for 1 h, then washed and incubated for an additional hour with [^{125}I]activin A (75,000 cpm) alone or together with heparan sulfate (Hep Sulf) (10 μg/ml) to remove cell-surface-associated FST. FST288 efficiently bound to the cell surface, whereas FS315 and FSTL3 binding was negligible. FST303 exhibited an intermediate cell-surface binding capacity. A representative experiment is shown. B, HepG2 cells were incubated with FST isoforms or FSTL3 (1 μg/ml) in the absence (*rows* A and C) or presence (*rows* B and D) of heparin sulfate (HS). *Rows* A and B are without and *rows* C and D are with permeabilization before incubation with FST or FSTL3. Bound proteins were detected with anti-Myc primary and fluorescent second antibodies. As expected from Fig. 3A, FST288 bound to a greater degree than FST303, whereas FST315 and FSTL3 binding was undetectable. After permeabilization, FST288 binding was substantially increased, and this binding was eliminated by heparin sulfate, indicating that FST288, but not the other isoforms, bound to intracellular membrane-associated proteoglycans.

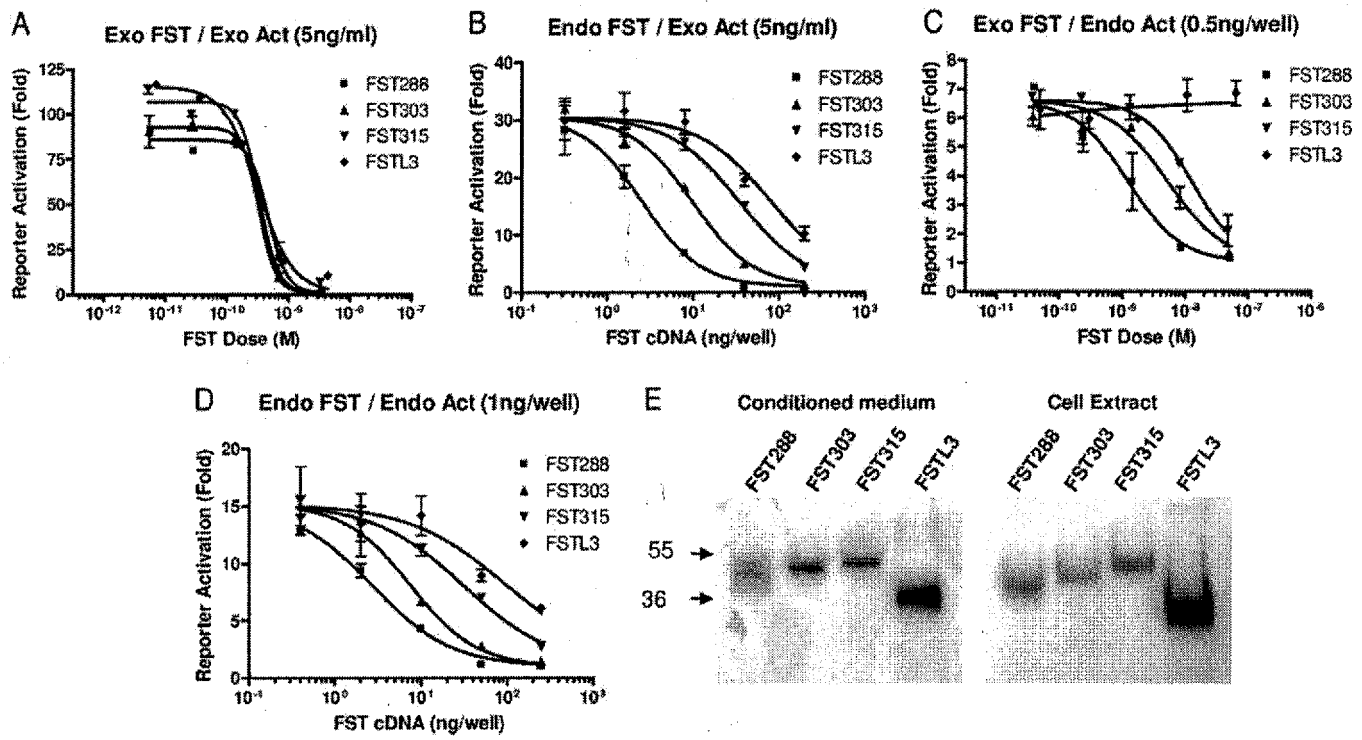

FIG. 4. Differential inhibition of exogenous *vs.* endogenous activin bioactivity by FST isoforms and FSTL3 in HepG2 cells. A, Increasing amounts of recombinant FST isoform or FSTL3 proteins were preincubated with 0.2 nM activin protein and then added to the cells. Binding proteins had equivalent inhibitory activity when both FST and activin were added exogenously (Exo). B, Cells were transfected with increasing amounts of FST/FSTL3 cDNA and then treated with 0.2 nM activin. When FST/FSTL3 proteins were made endogenously (Endo) but activin delivered exogenously (Exo), FST288 was most active, followed by FST303, FST315, and FSTL3, the order seen for proteoglycan binding in Fig. 3. C, Cells were transfected with 0.5 ng/well activin cDNA and then exogenously (Exo) treated with FST isoforms. When activin was endogenously (Endo) produced but FST/FSTL3 delivered exogenously, the same relative potency as in B was observed, but FSTL3 activity was undetectable. D, Both activin and FST/FSTL3 were transfected. When both activin and FST/FSTL3 were produced endogenously (Endo), all proteins were active in the same order as in B. Therefore, FST isoform capacity to inhibit endogenous activin correlates with cell-surface binding activity (FST288 > FST303 > FST315 > FSTL3). E, For experiments where FST isoforms or FSTL3 were transfected, cell extracts were analyzed by SDS-PAGE and Western blot to verify that equal amounts of protein were produced for each isoform. A representative experiment is shown.

isoforms and FSTL3 were added exogenously but the activin produced endogenously (Fig. 4C) as well as when both FST isoforms and FSTL3 along with activin were produced endogenously (Fig. 4D). To ensure that bioactivity differences were not a result of differential production of FST isoforms or FSTL3, we examined conditioned medium and extracts from transfected HepG2 cells (Fig. 5E). When analyzed by Western blot, all proteins were detected in both conditioned medium and cell extracts. Interestingly, FSTL3 was expressed to a greater degree than any of the other proteins, although it had the lowest bioactivity in Fig. 4, B–D. Conversely, FST288 was expressed at the lowest levels but was the most active. These results indicate that increased activity of FST288 was not because of superior protein biosynthesis. Thus, differential cell-surface binding among the FST isoforms and FSTL3 correlates with differential bioactivity when the FST/FSTL3, activin, or both are derived from endogenous sources, as would be expected when FST and/or activin are acting in an autocrine mode.

Differential modulation of activin-mediated proliferation by FST288 and FST315 in TT cells

The TT mouse testicular tumor cell line produces substantial levels of endogenous activin and depends on this activin for proliferation (34, 37), making these cells a more physiological *in vitro* model for comparing the ability of FST isoforms to differentially suppress activin-mediated proliferation. We evaluated the ability of FST288 and FST315, because they were the most distinct in suppressing activin signal transduction, to modulate TT cell proliferation by creating stably transfected cell lines. These stable cell lines express both FST and activin endogenously, which is critical because it was previously shown that exogenous FST288 treatments can only weakly inhibit activin-mediated cell growth in these cells (34, 37). The highest FST-secreting clones were selected using our free FST immunoassay, which ensured that in all cases, FST concentration in the medium was in excess of activin (data not shown). Compared with untransfected (wild-type) cells, proliferation in FST315-transfected TT cell lines was significantly enhanced (~25%; $P < 0.01$), whereas proliferation in FST288-transfected lines was suppressed by more than 50% ($P < 0.001$) (Fig. 5A).

To verify this differential activity in unselected cells, we generated adenoviruses expressing FST288 or FST315 under the control of the cytomegalovirus promoter and used them to infect different pools of wild-type TT cells derived from an identical stock. Free FST could easily be detected in conditioned medium of FST288 and FST315 virus-infected cells

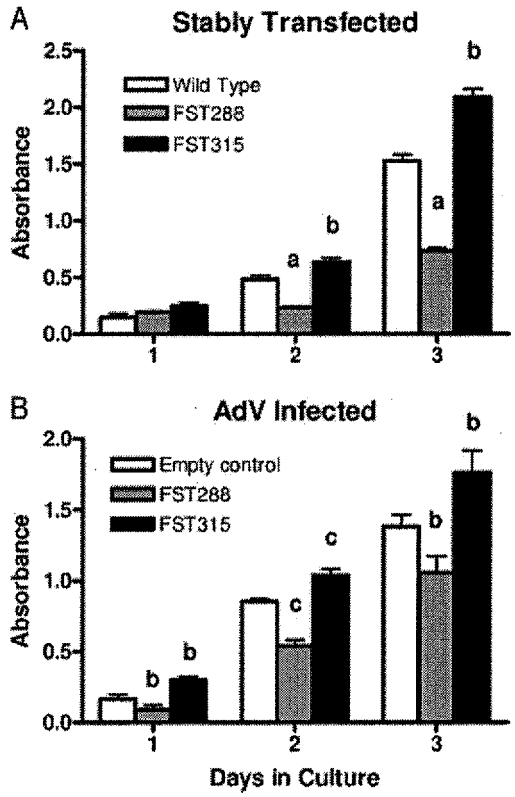

FIG. 5. Differential effect of FST288 *vs.* FST315 on TT cell proliferation. A, TT cells were transfected with FST288 or FST315 cDNA and stable colonies selected; B, Wild-type TT cells were infected with recombinant FST288, FST315, or empty adenoviruses. Cell proliferation was assessed using colorimetric proliferation assays. In both paradigms, FST288 significantly suppressed, whereas FST315 significantly stimulated, proliferation. a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$.

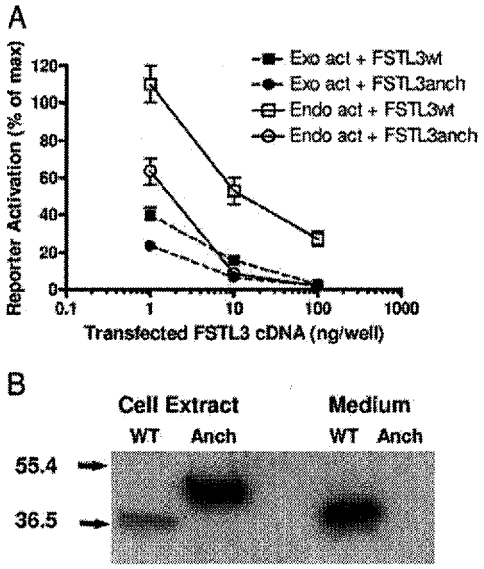

FIG. 6. Increased inhibition of endogenous activin of cell-surface-anchored FSTL3. A, FSTL3 was fused to the transmembrane domain of pDisplay and then transfected into HEK 293 cells together with the CAGA-luc reporter and βA cDNA [endogenous activin (Endo act)] or without activin βA cDNA. For exogenous activin (Exo act) wells, cells were treated with 0.2 nM activin. Cells transfected with wild-type (wt) FSTL3 cDNA were used as control. Results are graphed as percentage of maximum activin activity in the absence of FSTL3. Both wild-type and anchored (anch) FSTL3 (*squares* and *circles*, respectively) inhibited exogenous activin at all DNA doses (*dashed lines*) as expected from Fig. 4A. In contrast, anchored FSTL3 was nearly 10-fold more active (ED_{50} dose approximately 10-fold lower) than wild-type FSTL3 at inhibiting endogenous activin (*solid lines*), demonstrating that cell-surface localization by itself affords a substantial advantage for inhibiting endogenous activin. B, Cell extracts (10 μl) and conditioned medium (20 μl) from the 100 ng/well were analyzed by SDS-PAGE and Western blot. The vast majority of wild-type FSTL3 (WT) was found in the medium as expected, whereas anchored FSTL3 (Anch) was located only in the cellular fraction. In addition, the total FSTL3 protein for both fractions was quite similar, indicating that differences in activity were not because of differential expression. Results from a representative experiment are shown.

24 h after infection (54 and 76 ng/ml, respectively). As observed in the stable cell lines, expression of FST315 by viral infection significantly enhanced proliferation (~25%; $P < 0.05$), whereas FST288 significantly suppressed proliferation (25–40%; $P < 0.01$) (Fig. 5B). These observations demonstrate that FST288 and FST315 differentially regulate TT cell proliferation, likely reflecting their differential ability to suppress endogenous activin activity as described in Fig. 4.

Cell-surface binding of FSTL3 increases biological activity

To further test the hypothesis that cell-surface binding is responsible for the superior inhibition of endogenous activin by FST288, we expressed FSTL3 as a fusion protein with the transmembrane domain of the pDisplay vector, which anchors FSTL3 to the exterior surface of the plasma membrane (anchored FSTL3). When tested with exogenous activin, 1–10 ng of both wild-type and anchored FSTL3 suppressed more than 75% of activin's activity (Fig. 6A). However, 10 ng of wild-type FSTL3 suppressed less than 50% of endogenous activin activity, whereas the same amount of anchored FSTL3 suppressed 90%, reaching similar levels of activity as FST288. To verify that anchored FSTL3 was not secreted and thus must have been acting at the cell surface, we compared FSTL3 in cell extracts and conditioned medium by SDS-PAGE and Western blot. In cell extracts, both wild-type and anchored FSTL3 were detected whereas in conditioned medium, only wild-type FSTL3 was seen (Fig. 6B), and the protein concentration of wild-type FSTL3 in both compartments was similar to membrane-bound anchored FSTL3. Taken together, these results demonstrate that localization at the cell surface, as occurs for FST288 because of its HBS, and in anchored FSTL3, greatly enhances the ability of FST and FSTL3 to suppress endogenous activin activity.

FST isoforms and FSTL3 differentially bind related TGFβ-family ligands

FST isoforms and FSTL3 have been reported to bind myostatin (24, 25) and various BMPs (38), but isoform-specific differences in specificity have not been previously examined. Thus, we compared the ability of myostatin and BMPs to compete with radiolabeled activin for binding to the FST isoforms and FSTL3. Among the three FST isoforms, there was little difference in the binding potency of myostatin, BMP6, or BMP7 relative to activin, whereas BMP2 and BMP4

were inactive (Fig. 7, A–C, and Table 2). In contrast, whereas myostatin bound to FSTL3 at a relative potency comparable to the FST isoforms, BMP6 and BMP7 as well as BMP2 and BMP4 were inactive (Fig. 7D and Table 2). Thus, although there was little difference in TGFβ-family ligand specificity among the FST isoforms, BMP binding to FSTL3 was substantially reduced compared with FST isoforms.

To test the relevance of these relative binding activities on biological activity of TGFβ ligands, we examined the ability of exogenous FST isoforms and FSTL3 to inhibit the activity of exogenously added ligands in the HepG2 cell reporter assay. As observed earlier, and consistent with the binding results, the FST isoforms and FSTL3 had similar activin-inhibiting activity (Fig. 8A). In addition, neither the FST isoforms nor FSTL3 inhibited BMP2 or -4 activity (Fig. 8, B and C), and FSTL3 did not inhibit BMP6 and -7 bioactivity (Fig. 8, D and E). On the other hand, FST288 inhibited BMP6 and -7 bioactivity (Fig. 8, D and E) to a greater degree than the other FST isoforms despite little difference between the isoforms in the binding assay. Similarly, although the three FST isoforms were equally active in inhibiting myostatin bioactivity (Fig. 8F), FSTL3 had about 5-fold less inhibitory activity despite having similar binding activity. Taken together with the binding studies, these bioassay results demonstrate that differential binding specificity of the FST isoforms and FSTL3 for activin, BMPs, and myostatin cannot, by itself, account for differences in inhibition of TGFβ ligand biological activity.

Discussion

The three FST isoforms have different biochemical characteristics relating to the activity of their HBS through which they bind cell-surface proteoglycans, a property that also leads to isoform compartmentalization in different tissues (14–18). These distinctions suggest the possibility that the FST isoforms might be responsible for different subsets of the biological actions attributable to FST *in vivo*. To explore this possibility, we compared the activin-binding affinities and kinetics, cell-surface binding, ligand specificity, and biological activity of the isoforms to FSTL3, which unlike FST, does not contain an HBS and thus cannot bind to cell-surface proteoglycans (23). Our results demonstrate that the activin-binding affinity and kinetics of the FST isoforms and FSTL3 were not distinguishable. Moreover, the potencies of BMPs and myostatin for binding to the FST isoforms were not different, although BMP binding to FSTL3 was substantially reduced. However, the FST288 isoform, which was superior in cell-surface proteoglycan binding because of its uninhibited HBS, inhibited endogenous activin, myostatin, and BMP bioactivity to a greater degree compared with the other isoforms or FSTL3. Moreover, we found that FST288 suppressed, whereas FST315 enhanced, activin-mediated proliferation in TT cells. These observations indicate that the major biochemical parameter affecting biological activity among the isoforms and FSTL3 is the degree of cell-surface association. Additional support for this concept was obtained by expressing FSTL3 as a plasma membrane-anchored protein. In this context, the ability of anchored FSTL3 to inhibit endogenous activin was substantially increased rel-

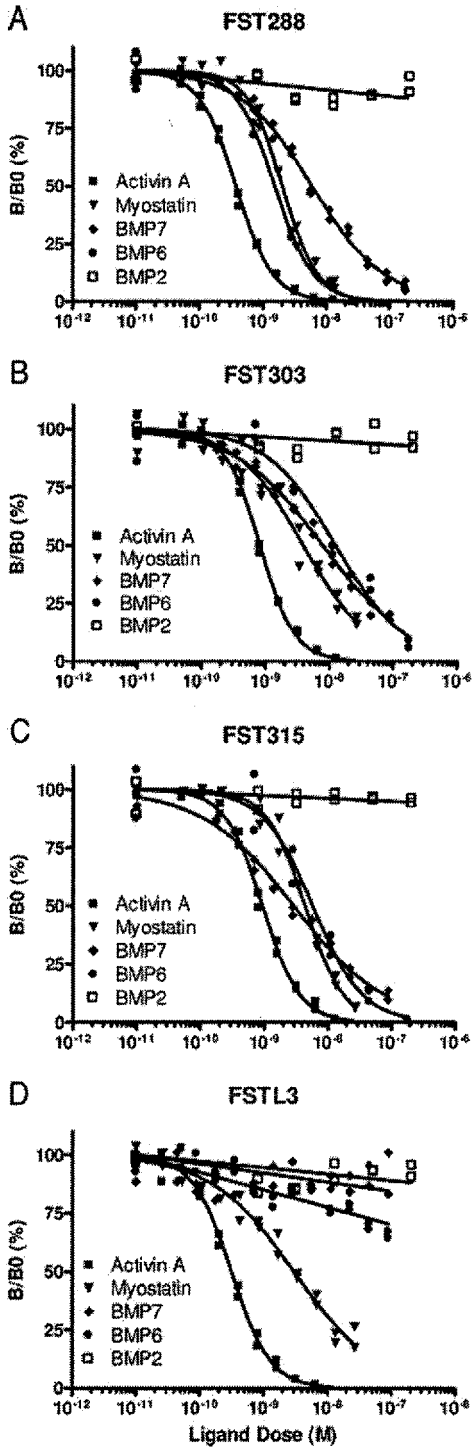

FIG. 7. Specificity of FST isoforms and FSTL3 for TGFβ family ligands. Purified FST proteins (25 ng/well) were attached to microtiter plates and then treated with radiolabeled activin A (75,000 cpm/well) together with increasing amounts of unlabeled activin A, myostatin, BMP7, BMP6, or BMP2. For the three FST isoforms (A–C), potency for competition with radiolabeled activin was as follows: activin A > BMP6 ≥ myostatin ≥ BMP7. BMP2 and BMP4 (not shown) were inactive. For FSTL3 (D), relative potency was activin A > myostatin, but there was no significant competition by BMP6, -7, -2, or -4 (not shown). A representative experiment of three to five determinations is shown.

TABLE 2. ED_{50} estimates for TGFβ superfamily members as competitors with radiolabeled activin

Ligand	FST288	FST303	FST315	FSTL3
Activin A	0.49 ± 0.09	1.37 ± 0.3	1.15 ± 0.3	0.34 ± 0.1
Myostatin	3.02 ± 0.08	2.46 ± 0.3	3.68 ± 0.9	1.53 ± 0.5
BMP7	5.45 ± 1.2	5.86 ± 0.8	14.3 ± 5.6[a]	NC
BMP6	1.24 ± 0.3	19.9 ± 12.5	3.88 ± 1.1	NC

FST isoforms and FSTL3 were passively adsorbed to solid-phase supports. Competition assays were performed by adding radiolabeled activin and increasing doses of unlabeled activin, myostatin, BMP7, or BMP6, and ED_{50} values were calculated. Results are mean ± SE (nM) for at least three independent experiments. NC, No curve.

[a] Estimate, slopes not parallel.

ative to soluble FSTL3, approximating the suppression afforded by FST288 itself. Our results therefore demonstrate that cell-surface association is the major biochemical determinant of biological potency in suppressing the bioactivity of endogenous activin and related ligands

Inhibition of activin and related TGFβ-family ligands by cell-surface-bound FST is analogous to inhibition of autocrine/paracrine actions of these ligands *in vivo*. Such situations might be encountered in tissue differentiation during embryonic development or in the neonate and adult in regulation of these ligands within tissues. In fact, the observations in TT cells are a model for autocrine/paracrine regulation of activin action because TT cells produce substantial amounts of activin. Thus, we found that endogenous FST288 was effective in inhibiting activin-dependent proliferation in TT cells, whereas FST315 was not. Although we did not test FSTL3 in this context, based on our results in the *in vitro* bioassays as well as previous studies (27), FSTL3 would be expected to be even weaker than FST315 in inhibiting autocrine activin. Nevertheless, when FSTL3 was anchored to the cell surface by a heterologous transmembrane sequence, its ability to inhibit endogenous activin was greatly enhanced. Our results are therefore consistent with the HBS and cell-surface binding being critical for maximal inhibition of endogenous activin and related ligands so that *in vivo*, FST288, and to a lesser degree, FST303, would be expected to serve these roles preferentially to FST315 and FSTL3. *In vivo* examination of this concept is underway.

The seemingly anomalous increase in TT cell proliferation when transfected or infected with FST315 vectors is more challenging to explain. However, it has been shown that increasing doses of activin resulted in receptor down-regulation in TT cells, suggesting that because these cells constitutively produce copious amounts of activin, their signaling system is chronically down-regulated (37). Although our results demonstrate that FST315 is a less effective inhibitor of

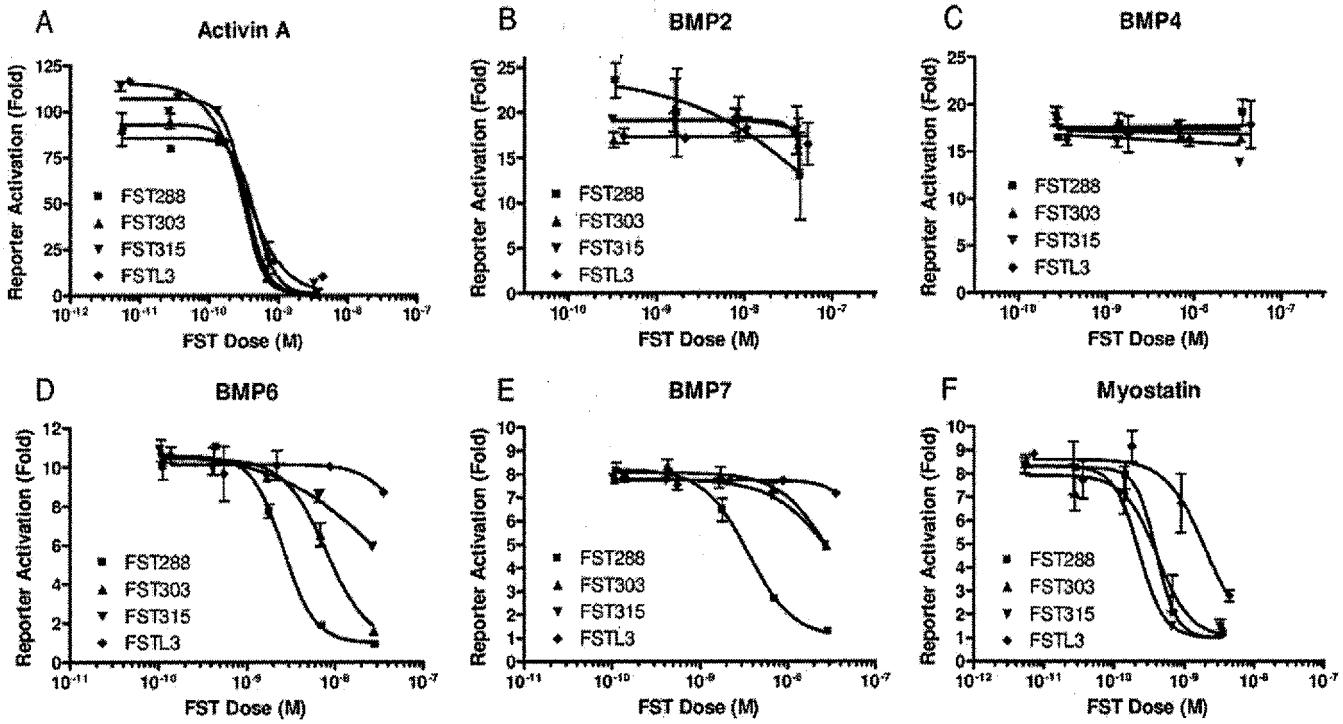

FIG. 8. Differential inhibition of exogenous TGFβ family biological activity by exogenous FST isoforms and FSTL3. HepG2 (activin and BMP assays) or HEK 293 (myostatin assays) cells were transfected with the CAGA-luc (activin and myostatin) or BRE-luc (BMPs) reporters and treated with TGFβ family ligands (activin and myostatin, 0.2 nM; BMP2 and -4, 0.4 nM; BMP6 and -7, 0.3 nM) together with increasing amounts of FST isoforms and FSTL3 as indicated in the figures. FST isoforms and FSTL3 inhibited activin (A) similarly, whereas they were all unable to inhibit BMP2 (B) and BMP4 (C). However, only FST288, and to a lesser degree FST303, significantly inhibited BMP6 (D), whereas only FST288 inhibited BMP7 (D). Inhibition of myostatin (F) was similar to activin except that FSTL3 was about 5-fold less active than FST isoforms.

endogenous activin compared with FST288, transfection with FST315 may still have inhibited activin activity sufficiently to reduce the level of receptor down-regulation, thereby resulting in a more sensitive activin signaling system. These more sensitive TT cells might then respond to the remaining endogenous activin with enhanced proliferation.

This situation in TT cells is somewhat analogous to the pituitary where activin is produced endogenously and constitutively and is regulated by FST (4, 39–41). It is not presently known which FST isoform is produced within the pituitary, although there is some suggestion that it might be FST315 (42, 43). Thus, one possible mode of FST315 regulation of activin-stimulated FSH biosynthesis in the pituitary may be through reducing down-regulation of gonadotroph activin receptors, thereby producing greater activin sensitivity and FSH release.

In contrast to inhibition of endogenous activin, exogenous FST isoforms and FSTL3 were equally effective at inhibiting exogenous activin. This is equivalent to the *in vivo* situation of inhibiting activin and related ligands in the circulation. Moreover, to maintain effective serum concentrations of FST and FSTL3, isoforms that do not bind cell-surface proteoglycans would be preferable. Consistent with differential distribution of FST isoforms within the body, we recently identified FST315 as the circulating FST isoform in humans (18), whereas FSTL3, but not FST, was identified as a circulating binding protein for myostatin (24). Thus, our results indicate that the distribution of FST isoforms correlates with their heparin-binding activity so that FST315 and FSTL3 act primarily as regulators of endocrine activin, myostatin, and perhaps BMPs. Moreover, our results are consistent with a compartmentalization of FST isoforms based on cell-surface binding that results in their being responsible for different biological actions *in vivo*.

In addition to the classical signaling pathway of activin binding to cell-surface receptors and transducing an intracellular signaling cascade, recent evidence has been presented for another pathway where activin or TGFβ, bound to type II receptor, is endocytosed to early endosomes. In this model, activin or TGFβ signals are transduced via contact with Smad anchor for receptor activation (SARA)-bound Smad proteins that reside in endosomes (44–46). Within this system, FST could act at the cell surface to antagonize activin binding or enter the cell via endocytosis along with the activin-receptor complex where it could then reduce signaling by sequestering activin as it dissociates from receptors. FST288 has also been reported to bind radiolabeled activin at the cell surface, where it is then internalized and presumably degraded (47). Thus, FST288, by virtue of its greater ability to bind cell-surface proteoglycans, may have greater activin-inhibitory activity by antagonizing activin both at the cell surface and within endosomes, whereas FST315 and FSTL3 can act only on activin that is extracellular. Intracellular localization and possibly activity of FST288 is supported by the immunocytochemical results in this study showing FST288 binding to intracellular membranes that is reversible by heparin treatment, as well as by our previous results demonstrating that some newly synthesized FST288 remains in the cytoplasm for up to 4 h (48). Regardless of where FST288 actually inhibits activin binding to its receptor, it clearly has increased ability to inhibit endogenous activin activity relative to the other isoforms and FSTL3. These results collectively indicate that the different FST isoforms may have different *in vivo* biological roles and that FST315 will have activities more closely related to FSTL3 because both have low cell-surface binding ability.

Although high-affinity binding of FST to activin and myostatin has been previously reported (21, 25, 31, 36, 49), the relative activity of the three FST isoforms, as directly compared here, has not been described. In addition, there is some disagreement over relative binding affinities for the different ligands owing to the wide range of different techniques used (36, 49, 50). For example, different dissociation constants were reported for FST288 and FST315 binding to activin immobilized on a Biacore chip (36), which contrasts with our determination of similar affinities among the FST isoforms. However, the recently reported crystal structure for the FST288-activin complex (51), which found two FST molecules bound to one activin dimer, appeared to require flexibility of the activin dimer because its configuration in complex with FST was distinct from its conformation in complex with its receptor (52). Thus, immobilization of activin during surface plasmon resonance analysis with the Biacore system may inhibit this flexibility, leaving activin in an unnatural conformation that favored one FST isoform over another. This may also be applicable to previously reported affinities for FST binding to BMP4 determined using the same technology (50). In our study, activin was presented in solution and may thus be more physiological because *in vivo*, TGFβ ligands are more likely to contact FST or FSTL3 in solution. Nevertheless, our results indicate that when compared directly in competition assays as in this study, activin appeared to be the preferred ligand for FST isoforms and FSTL3.

FSTL3 has been identified as a circulating binding protein for myostatin in humans and mice (24). However, these investigators were unable to identify any circulating myostatin bound to FST using SDS-PAGE followed by mass spectroscopy. This is unexpected because the FST isoforms appear to neutralize myostatin better than FSTL3. We have previously reported (21, 31), and confirmed here, that activin binding to FST is essentially irreversible, as appears to be the case for myostatin as well (49). Moreover, FST has been shown to inhibit myostatin activity when expressed transgenically in muscle (25). Taken together, these studies indicate that both FST and FSTL3 can bind and neutralize activin and myostatin. The preferred ligand may depend on which ligand gains access first to either FST or FSTL3, which is in turn a function of differential tissue or organ distribution of FST and FSTL3 (21).

In summary, our results clarify the activin-binding affinity among the FST isoforms and FSTL3 and demonstrate that their differential activin-regulating activity is dependent on their relative cell-surface binding activity rather than on differential activin-binding affinity. Our results also define the relative specificity of binding and inhibitory activity for a number of related TGFβ-family ligands by the FST isoforms and FSTL3, with FSTL3 being almost completely inactive in regulating BMP ligands, thereby suggesting that *in vivo*, FSTL3 is unlikely to regulate BMP activity. Finally, our results suggest that the *in vivo* biological roles of the FST iso-

forms and FSTL3 are likely to be distinct, dependent on their relative cell-surface binding activity and consequent compartmentalization within the body as well as on colocalization of biosynthesis in different tissues.

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References

1. **Welt C, Sidis Y, Keutmann H, Schneyer A** 2002 Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. Exp Biol Med (Maywood) 227:724–752
2. **Phillips DJ** 2000 Regulation of activin's access to the cell: why is mother nature such a control freak? Bioessays 22:689–696
3. **DePaulo LV, Bicsak TA, Erickson GF, Shimasaki S, Ling N** 1991 Follistatin and activin: a potential intrinsic regulatory system within diverse tissues. Soc Exp Med Biol 200:500–512
4. **Besecke LM, Guendner MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC, Weiss J** 1997 Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle. Endocrinology 138:2841–2848
5. **Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM** 2003 Granulosa cell-specific inactivation of follistatin causes female fertility defects. Mol Endocrinol 18:953–967
6. **Guo Q, Kumar TR, Woodruff TW, Hadsell LA, DeMayo FJ, Matzuk MM** 1998 Overexpression of mouse follistatin causes reproductive defects in transgenic mice. Mol Endocrinol 12:96–106
7. **Takabe K, Wang L, Leal AM, MacConell LA, Wiater E, Tomiya T, Ohno A, Verma IM, Vale W** 2003 Adenovirus-mediated overexpression of follistatin enlarges intact liver of adult rats. Hepatology 38:1107–1115
8. **Wankell M, Munz B, Hubner G, Hans W, Wolf E, Goppelt A, Werner S** 2001 Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. EMBO J 20:5361–5372
9. **Jones KL, Kretser DM, Patella S, Phillips DJ** 2004 Activin A and follistatin in systemic inflammation. Mol Cell Endocrinol 225:119–125
10. **Matzuk MM, Lu N, Vogel HJ, Sellheyer K, Roop DR, Bradley A** 1995 Multiple defects and perinatal death in mice deficient in follistatin. Nature 374:360–363
11. **Yao HH, Matzuk MM, Jorgez CJ, Menke DB, Page DC, Swain A, Capel B** 2004 Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. Dev Dyn 230:210–215
12. **Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, Koba A, Ueno N, Ying SY, Ling N, Guillemin R** 1988 Primary structure of the human follistatin precursor and its genomic organization. Proc Natl Acad Sci USA 85:4218–4222
13. **Sugino K, Kurosawa N, Nakamura T, Takio K, Shimasaki S, Ling N, Titani K, Sugino H** 1993 Molecular heterogeneity of follistatin, an activin-binding protein. J Biol Chem 68:15579–15587
14. **Sumitomo S, Inouye S, Liu XJ, Ling N, Shimasaki S** 1995 The heparin binding site of follistatin is involved in its interaction with activin. Biochem Biophys Res Commun 208:1–9
15. **Inouye S, Ling N, Shimasaki S** 1992 Localization of the heparin binding site of follistatin. Mol Cell Endocrinol 90:1–6
16. **Nakamura T, Sugino K, Titani K, Sugino H** 1991 Follistatin, an activin-binding protein, associates with heparan sulfate chains of proteoglycans on follicular granulosa cells. J Biol Chem 266:19432–19437
17. **Sidis Y, Schneyer AL, Keutmann HT** 2005 Heparin and activin-binding determinants in follistatin and FSTL3. Endocrinology 146:130–136
18. **Schneyer AL, Wang Q, Sidis Y, Sluss PM** 2004 Differential distribution of follistatin isoforms: application of a new FS315-specific immunoassay. J Clin Endocrinol Metab 89:5067–5075
19. **Hayette S, Gadoux M, Martel S, Bertrand S, Tigaud I, Magaud JP, Rimokh R** 1998 FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. Oncogene 16:2949–2954
20. **Schneyer A, Tortoriello D, Sidis Y, Keutmann H, Matsuzaki T, Holmes W** 2001 Follistatin-related protein (FSRP): a new member of the follistatin gene family. Mol Cell Endocrinol 180:33–38
21. **Tortoriello DV, Sidis Y, Holtzman DA, Holmes WE, Schneyer AL** 2001 Human follistatin-related protein: a structural homologue of follistatin with nuclear localization. Endocrinology 142:3426–3434
22. **Xia Y, Sidis Y, Schneyer A** 2004 Overexpression of follistatin-like 3 in gonads causes defects in gonadal development and function in transgenic mice. Mol Endocrinol 18:979–994
23. **Sidis Y, Tortoriello DV, Holmes WE, Pan Y, Keutmann HT, Schneyer AL** 2002 Follistatin-related protein and follistatin differentially neutralize endogenous *vs.* exogenous activin. Endocrinology 143:1613–1624
24. **Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y** 2002 The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277:40735–40741
25. **Lee SJ, McPherron AC** 2001 Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98:9306–9311
26. **Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, Sugino H** 2000 Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-β family. J Biol Chem 275:40788–40796
27. **Otsuka F, Moore RK, Iemura S, Ueno N, Shimasaki S** 2001 Follistatin inhibits the function of the oocyte-derived factor BMP-15. Biochem Biophys Res Commun 289:961–966
28. **Keutmann HT, Schneyer AL, Sidis Y** 2004 The role of follistatin domains in follistatin biological action. Mol Endocrinol 18:228–240
29. **McConnell DS, Wang QF, Sluss PM, Bolf N, Khoury RH, Schneyer AL, Midgley Jr. AR, Reame NE, Crowley Jr WF, Padmanabhan V** 1998 A two-site chemiluminescent assay for activin-free follistatin reveals that most follistatin circulating in men and normal cycling women is in an activin-bound state. J Clin Endocrinol Metab 83:851–858
30. **Bernstein JR, Crowley Jr WF, Schneyer AL** 1990 An improved method of purifying inhibin radioligand for radioimmunoassay. Biol Reprod 43:492–496
31. **Schneyer AL, Rzucidlo DA, Sluss PM, Crowley Jr WF** 1994 Characterization of unique binding kinetics of follistatin and activin or inhibin in serum. Endocrinology 135:667–674
32. **Korchynskyi O, Ten Dijke P** 2002 Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891
33. **Dennler S, Itoh S, Vivien D, Ten Dijke P, Huet S, Gauthier JM** 1998 Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 17:3091–3100
34. **Shikone T, Matzuk MM, Perlas E, Finegold MJ, Lewis KA, Vale W, Bradley A, Hsueh AJW** 1994 Characterization of gonadal sex cord-stromal tumor cell lines from inhibin α and p53-deficient mice: the role of activin as an autocrine growth factor. Mol Endocrinol 8:983–995
35. **Wang X, Zeng W, Murakawa M, Freeman MW, Seed B** 2000 Episomal segregation of the adenovirus enhancer sequence by conditional genome rearrangement abrogates late viral gene expression. J Virol 74:11296–11303
36. **Hashimoto O, Kawasaki N, Tsuchida K, Shimasaki S, Hayakawa T, Sugino H** 2000 Difference between follistatin isoforms in the inhibition of activin signalling: activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. Cell Signal 12:565–571
37. **Di Simone N, Hall HA, Welt C, Schneyer AL** 1998 Activin regulates βA-subunit and activin receptor messenger ribonucleic acid and cellular proliferation in activin-responsive testicular tumor cells. Endocrinology 139:1147–1155
38. **Shimasaki S, Moore RK, Otsuka F, Erickson GF** 2004 The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101
39. **Bilezikjian LM, Corrigan AZ, Blount AL, Vale WW** 1996 Pituitary follistatin and inhibin subunit messenger ribonucleic acid levels are differentially regulated by local and hormonal factors. Endocrinology 137:4277–4284
40. **Corrigan AZ, Bilezikjian LM, Carroll RS, Bald LN, Schmelzer CH, Fendly BM, Mason AJ, Chin WW, Schwall RH, Vale W** 1991 Evidence for an autocrine role of activin B within rat anterior pituitary cultures. Endocrinology 128:1682–1684
41. **Bauer-Dantoin AC, Weiss J, Jameson JL** 1996 Gonadotropin-releasing hormone regulation of pituitary follistatin gene expression during the primary follicle-stimulating hormone surge. Endocrinology 137:1634–1639
42. **Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J** 1996 Gonadotropin-releasing hormone regulates follicle-stimulating hormone-β gene expression through an activin/follistatin autocrine or paracrine loop. Endocrinology 137:3667–3673
43. **Schneyer AL, Wang QF, Weiss J, Boepple P, Hall J, Khoury R, Taylor A, Pralong F, Sluss P, Crowley WF** 1997 Follistatin physiology and potential mechanisms of action in the human. In: Aono T, Sugino H, Vale WW, eds. Inhibin, activin and follistatin: regulatory functions in system and cell biology. New York: Springer-Verlag; 28–38
44. **Lin HK, Bergmann S, Pandolfi PP** 2004 Cytoplasmic PML function in TGF-β signalling. Nature 431:205–211

45. **Panopoulou E, Gillooly DJ, Wrana JL, Zerial M, Stenmark H, Murphy C, Fotsis T** 2002 Early endosomal regulation of Smad-dependent signaling in endothelial cells. J Biol Chem 277:18046–18052
46. **Itoh F, Divecha N, Brocks L, Oomen L, Janssen H, Calafat J, Itoh S, Dijke PP** 2002 The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-β/Smad signalling. Genes Cells 7:321–331
47. **Hashimoto O, Nakamura T, Shoji H, Shimasaki S, Hayashi Y, Sugino S** 1997 A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. J Biol Chem 272:13835–13842
48. **Saito S, Sidis Y, Mukherjee A, Xia Y, Schneyer A** 2005 Differential biosynthesis and intracellular transport of follistatin isoforms and follistatin-like-3 (fstl3). Endocrinology 146:5052–5062
49. **Amthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K** 2004 Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis. Dev Biol 270:19–30
50. **Iemura I, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N** 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. Proc Natl Acad Sci USA 95:9337–9342
51. **Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS** 2005 The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. Dev Cell 9:535–543
52. **Thompson TB, Woodruff TK, Jardetzky TS** 2003 Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-β ligand:receptor interactions. EMBO J 22:1555–1566

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